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SAFETY AND IMMUNOGENICITY OF A LIVE-ATTENUATED JUNIN (ARGENTINE HEMORRHAGIC FEVER) VACCINE IN RHESUS MACAQUES

KELLY T. McKEE JR., JULIO G. BARRERA ORO, ANNA I. KUEHNE, JOAN A. SPISSO, AND BILL G. MAHLANDT

U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland

Abstract. The safety and immunogenicity of Candid #1, a live-attenuated Junin virus vaccine, were evaluated in rhesus macaques. Candid #1 was inoculated subcutaneously in graded doses ranging from 16 to 127,200 plaque-forming units (PFU) into four groups of five animals each; four controls received saline. There was no significant effect of the immunization on any physical, hematologic, or biochemical parameter measured. Junin virus was recovered by cocultivation from peripheral blood mononuclear cells (PBMC) of 14 (70%) of 20 animals from 1 to 21 days after immunization; 27 (12%) of 223 PBMC samples that represented animals in all four dose groups were positive. In contrast, virus was recovered from the plasma of only two of 20 macaques (two of 225 samples [0.9%]), and only once (by amplification) from throat swabs. No evidence of reversion was detected in any blood isolate. All animals developed a detectable neutralizing antibody response following vaccination. These results indicate that Candid #1 is safe and immunogenic in nonhuman primates.

Argentine hemorrhagic fever (AHF) is a potentially lethal infection of humans recognized among the inhabitants of the small, but economically important, humid pampas of northcentral Argentina. The etiologic agent, Junin virus, is a member of the family *Arenaviridae*. The virus is maintained in nature through chronic carriage in several species of cricetine rodent, primarily *Calomys musculinus* and *C. laucha*.[1–3] Contamination with secreta and excreta of reservoir rodents, through abrasions or inhalation of highly infectious aerosols, constitutes the principal means by which human infection occurs.[4]

Untreated, mortality from AHF may approach 30% (Instituto Nacional de Estudios Sobre Virosis Hemorragicas, Pergamino, Argentina, unpublished data). Effective public health programs, together with early administration of immune plasma obtained from convalescent donors, have effectively reduced the death rate to less than 1%.[5–7] However, immunization of the at-risk population offers the only practical solution for control of the disease.

Attempts to develop a safe and effective AHF vaccine have been continuous since the late 1950s; none of these efforts has produced a product suitable for widespread human use.[8] Recently, a candidate live-attenuated immunogen was developed using serial suckling mouse brain (SMB) and cell culture passage of prototype XJ strain Junin virus. This vaccine, Candid #1, which was found to be phenotypically stable after in vitro passage, was significantly more attenuated for newborn mice and guinea pigs than XJ Clone 3 (an early generation human vaccine), and protected guinea pigs against lethal challenge with virulent Junin and Machupo virus strains. In addition, preliminary neurovirulence testing indicated that this vaccine was safe by intracerebral inoculation of rhesus macaques[9] (Barrera Oro J, Eddy G, unpublished data).

The current study documents the safety and immunogenicity of Candid #1 when administered by peripheral (subcutaneous) inoculation in rhesus macaques. These non-human primates have been previously shown to develop clinical syndromes following infection with wild-type strains that closely mimic human AHF.[10,11]

MATERIALS AND METHODS

Vaccine strain

Candid #1 vaccine was derived from an attenuated SMB passage of prototype XJ strain Junin virus by single-burst selection in certified fetal rhesus lung cells[12] (Barrera Oro J, Eddy G, unpublished data).

TABLE 1

*Group assignments for rhesus macaques inoculated with Candid #1 Junin vaccine**

Vaccine group	n	Weight (kg)†	Inoculum (PFU)	Volume (ml)	Route
1	5	9.0 (6.1–11.5)	16	0.5	SC
2	5	9.5 (7.1–12.4)	318	0.5	SC
3	5	10.1 (7.5–12.2)	6,360	0.5	SC
4	5	8.8 (5.7–11.4)	127,200	0.5	SC
5	4	9.8 (8.3–12.6)	Saline	0.5	SC

* PFU = plaque-forming units, SC = subcutaneous.
† Values are the mean (range).

Animal manipulations

Twenty four healthy adult *Macaca mulatta* (18 males and six females) weighing 5.7–12.4 kg were obtained from the primate colony at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) (Fort Detrick, Frederick, MD) (Table 1). Animals were housed individually in stainless steel cages with collapsibie backs. Their diet consisted of monkey chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. All animals were housed in a single room under Biosafety Level-3 (BL-3) containment.

Macaques were sedated with ketamine hydrochloride (7 mg/kg/dose) to obtain blood and throat swabs, and for physical examination. Blood specimens were obtained from saphenous or femoral veins using a 23- or 21-gauge butterfly needle. Venipuncture sites were cleansed with 70% ethanol before needle insertion.

In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee of Laboratory Animal Resources of the National Research Council. The facilities are fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Experimental design

Animals were assigned randomly to one of five experimental groups receiving 20-fold dilutions of Candid #1 or placebo (Table 1). On day 0, each macaque received 0.5 ml of vaccine or saline placebo by subcutaneous injection, according to group assignment.

Macaques were observed at least once a day for 106 days for signs of clinical illness. Objective disease parameters were monitored three times a week for four weeks after vaccination, and at monthly intervals thereafter. Objective parameters included physical examination, body weight, viremia, virus shedding from the oropharynx, neutralizing antibodies, clinical chemistry studies, and clinical hematology studies.

Blood and throat swabs were processed for virus and antibody determinations as described previously.[10] Clinical laboratory studies were performed under BL-3 conditions inside a specially designed biocontainment clinical laboratory.

Prior to termination of the study, all animals were challenged with virulent Junin virus to assess vaccine efficacy (McKee K Jr and others, unpublished data).

Infectivity assays

Materials for direct quantitative viral titration were assayed by plaquing the virus on Vero cell monolayers, as previously described.[13]

Peripheral blood mononuclear cells (PBMC) were cocultivated with Vero cell monolayers to facilitate virus recovery. Heparinized whole blood samples were centrifuged, the plasma was decanted, and the cellular elements were layered onto a cushion of histopaque 1077 (Sigma, St. Louis, MO) for separation. After the samples were centrifuged at 1,600 × *g* for 20 min, the visible mononuclear fraction was identified and withdrawn with a pasteur pipette, washed, and resuspended in RPMI 1640 (complete) medium. Each of two T-25 flasks containing confluent Vero cell monolayers was then inoculated with 5 ml of mononuclear cell suspension, and incubated at 36°C for one week. On the seventh day, the supernatants were decanted, and the cells were removed by shaking with glass beads. The cells were resuspended in saline, spotted onto slides, stained with a fluorescein isothiocyante–conjugated rabbit anti-Junin virus antiserum, and were examined by direct immunofluorescence. Decanted supernatants were combined and centri-

fuged, and the resulting cell pellet was resuspended in medium for application onto additional Vero monolayers in duplicate T-25 flasks. The supernatant fluid obtained from this centrifugation was assayed directly by plaquing on Vero cells as described above. All samples were passaged blindly in this manner through four cycles (unless interrupted by contamination) before being considered negative for the presence of virus.

Characterization of virus isolates

Selected isolates from the blood of vaccinated animals were identified as Junin virus by neutralization with a standard monkey serum. This serum (titer = 1:4,096) was collected from a rhesus macaque participating in a separate experiment who received a single inoculation with Candid #1 strain of Junin virus. For neutralizing virus isolates in the current study, the serum was diluted 1:5.

The potential for in vivo reversion of Candid #1 was assessed by examining the virulence of selected PBMC isolates for juvenile mice.[9,14,15] Briefly, 11–12-day-old female CD-1 strain mice (Charles River Breeding Laboratories, Charles River, MA) were inoculated intracerebrally with serial 10-fold dilutions of virus isolates obtained by cocultivation of PBMC or control virus strains (0.02 ml/dose). Each isolate also was titrated simultaneously by a plaque assay in Vero cells as described above. All inocula were used at similar low (1–2) cell passage levels. Animals were observed daily for 21 days, and 50% lethal dose (LD_{50}) values were determined by the method of Reed and Muench.[16] A Lethal Index (LI) was then was calculated according to the following formula: $LI = (\log_{10}PFU) - (\log_{10}LD_{50})$, where PFU = the number of plaque-forming units obtained by Vero cell plaque assay.

Virus strains used for comparison in this assay included the Candid #1 vaccine given to macaques and the XJ Clone 3 (SMB passage 1) attenuated Junin virus strain.

Serology

Neutralizing antibodies were measured against XJ Clone 3 Junin virus by a constant virus, serum dilution, plaque reduction technique as previously described.[11,17] Endpoints were recorded as the highest dilution yielding ≥ 80% reduction in plaque number.

Clinical laboratory studies

Venous blood was collected in glass tubes containing EDTA or heparin for hematology determinations. White blood cell counts, differential white blood cell counts, and platelet counts were obtained using an Ortho ELT-7 hematology analyzer (Ortho Diagnostics, Braintree, MA).

Serum biochemical values were measured with an Abbott VP analyzer (Abbott Laboratories, North Chicago, IL). Assay systems using kinetic (rate)-type reactions with commercially available kits were adapted for use on the Abbott analyzer.

Statistical analysis

Data were analyzed on a microcomputer at the Biometrics and Information Management Division (USAMRIID) using general linear models (GLM) programs available on SAS (Statistical Analysis System, Cary, NC) software. Differences among vaccinated groups over time, as well as differences between vaccinated groups and placebo controls, were determined by repeated measures analysis of variance procedures.

RESULTS

Clinical findings

Following inoculation of Candid #1 or saline placebo, all macaques remained generally healthy. No evidence of malaise, lethargy, diarrhea, constipation, rash, or mucous membrane hemorrhage was found in any animal. Depressed appetite was noted by a caretaker on days 37 and 39 postvaccination in one macaque who had received 6,360 PFU of Candid #1; food and water intake was unremarkable on all other days, and the relationship of this isolated event to immunization is unclear. Appetites were normal in all other macaques throughout the study.

Mean body weights decreased slightly from baseline levels in all groups (including placebo) during the initial 10 days after vaccination, possibly as a function of their having been frequently manipulated (Figure 1A). The weights then increased in all groups, although to a lesser degree in the animals receiving the two highest vaccine doses. However, in no case was weight loss observed to be of any physiologic significance. Rectal temperatures varied widely, and were higher

in all groups (including placebo) after vaccination (Figure 1B); however, the magnitude of this change was not statistically or biologically significant, and perhaps was another reflection of the extensive manipulation of these animals.

Clinical laboratory studies

Levels of the following parameters were determined in sequential serum samples obtained from all animals before and after immunization with Candid #1 or placebo: sodium, potassium, chloride, total protein, albumin, glucose, urea nitrogen, creatinine, calcium, magnesium, cholesterol, triglycerides, total bilirubin, amylase, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, alpha hydroxybutyrate dehydrogenase, gamma-glutamyl transpeptidase, lactate dehydrogenase, creatine kinase, total IgG, IgM, and IgA; and complement factors 3 and 4. Only four of the 26 parameters measured (sodium, potassium, urea nitrogen, and IgM) exhibited overall treatment effects by GLM repeated measures analysis. In all cases, observed effects could be discounted as normal variation (compared with pooled control and day 0 values), prevaccination differences (significant differences were observed only for day 0 values), or possible laboratory error (lower IgM in the group receiving 318 PFU of Candid #1 than in the group receiving 16 PFU).

Samples for clinical hematologic analysis were inadvertently collected into tubes containing heparin instead of EDTA for the first two days on which specimens were obtained (days 0 and 4). Although total white blood cell, absolute neutrophil, and platelet counts were mildly decreased on these two days when compared with subsequent values, statistically significant differences were seen only for neutrophils ($P = 0.02$, by repeated measures analysis of variance); absolute lymphocyte counts and packed cell volumes (hematocrit) were not affected. No other significant effects were observed for the remainder of the 28-day sampling period.

Virus isolation

Recovery of virus from blood by cocultivation of PBMC was attempted in all animals after immunization (Table 2). Twenty-seven Candid #1 isolates were obtained from 223 PBMC samples (12% positive). Isolates were recovered from 14 (70%) of 20 vaccinated macaques between one and 21 days after immunization; 23 (85%) of the 27 were made between days 4 and 14. In the group receiving the highest vaccine dose (127,200 PFU), isolations were made from three consecutive bleeds over one week in three of five animals, from two consecutive bleeds in one animal, and on a single occasion in the remaining macaque. Repeated isolations from the same animal were made once in the group receiving 6,360 PFU, and twice in each of the two groups receiving lower doses. In contrast, Candid #1 could be recovered only twice from 225 plasma samples (0.9%) by direct plaquing during the same period (Table 2). Both plasma isolates were obtained from two animals in whom PBMC were simultaneously positive for virus. Neither plasma- nor PBMC-associated viremia was observed in the presence of neutralizing antibody in any macaque at any time.

Virus could not be recovered by direct plaquing of oropharyngeal swabs obtained at the time animals were bled. However, amplification of cultures on Vero cell monolayers overlaid with liquid media for one week yielded a small number of plaques (titer = 1.68 $\log_{10}$ PFU/ml) on assay of culture supernatants from the day 10–sample of one macaque. This animal was a member of the group receiving the highest concentration of vaccine (127,200 PFU), and Junin virus was recovered from PBMC, but not from plasma, on the same day. No additional isolations were made by amplification of specimens from any other animals on any other days.

Characterization of isolates

Neutralization of 24 (89%) of 27 viruses recovered by cocultivation of PBMC, and both plasma isolates, was performed against a standard Candid #1 immune monkey serum. In all but four cases, complete neutralization (from 2.49 to 6.42 $\log_{10}$ PFU) was accomplished; in the remainder, the reduction in titer was > 99.95% (4.83–5.32 $\log_{10}$ PFU neutralized) (Table 3).

Eleven of the 27 PBMC isolates that were collected from animals 7–21 days after immunization and were representative of each vaccination group were evaluated for their virulence in 11–12-day-old juvenile mice (Table 4). Five isolates were less virulent than the parent vaccine (LI > 3.62), and all were less virulent than the

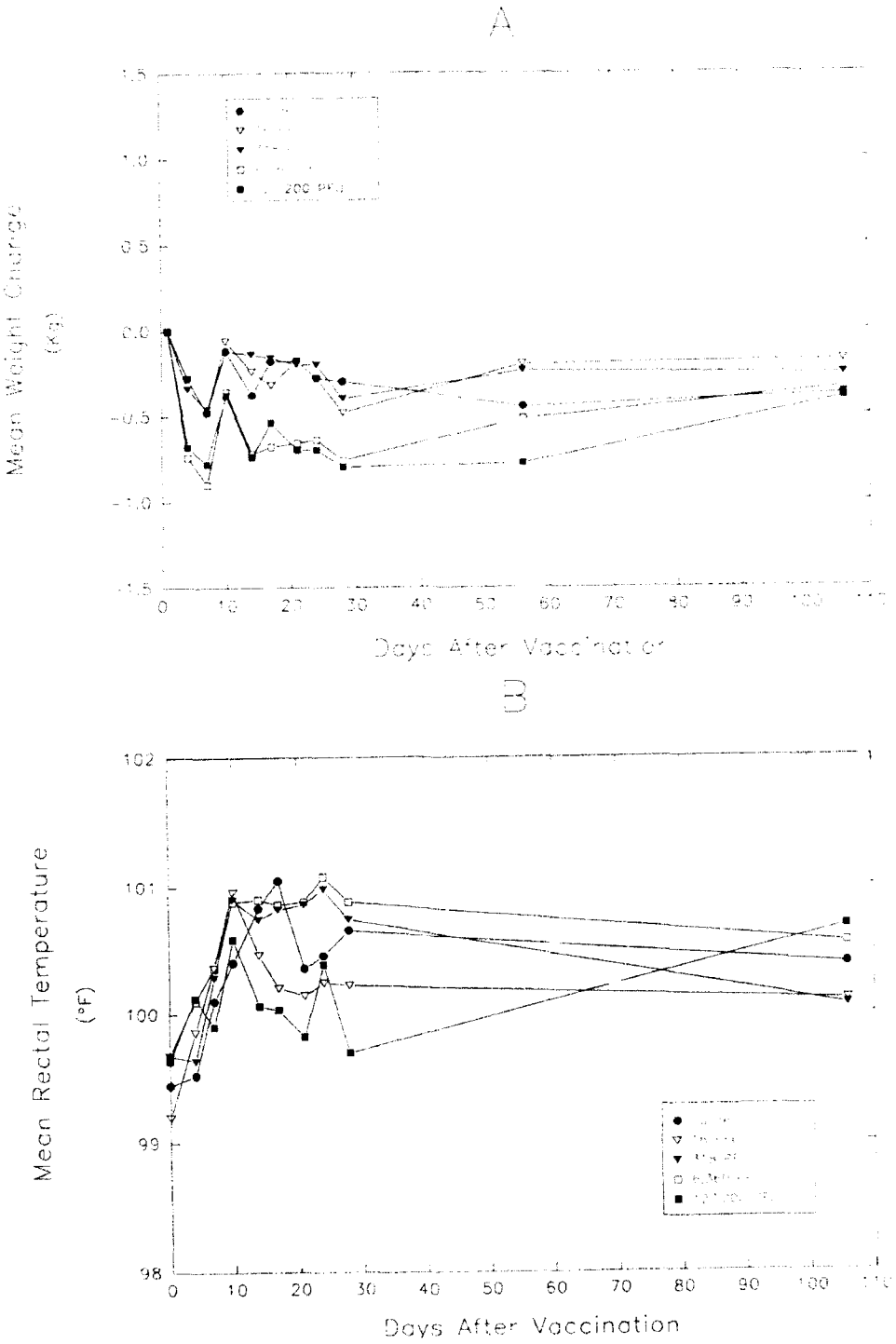

FIGURE 1. **A**, mean body weight change from baseline levels (adjusted to 0) in rhesus macaques inoculated with graded doses of Candid #1 vaccine or saline placebo. **B**, mean rectal temperature readings from rhesus macaques inoculated with Candid #1 or placebo. Error bars are omitted for clarity. No significant differences were detected between groups, or within groups over time. PFU = plaque-forming units.

TABLE 2

*Recovery of Junin virus by cocultivation of peripheral blood mononuclear cells in rhesus macaques inoculated with Candid #1 Junin vaccine**

Vaccine group	Day after vaccination											
	0	1	4	7	10	14	17	21	24	28	56	106
16 PFU	ND	0/5	0/5	0/5	2/5	2/4	0/4	1/5	0/5	0/5	0/5	0/5
318 PFU	ND	0/5	1/5†	2/5	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6,360 PFU	ND	0/5	1/5	1/5	1/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5
127,200 PFU	ND	1/5	2/5	4/5†	3/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5
Placebo	ND	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Total no. positive	0	1	4	7	8	4	2	1	0	0	0	0

* Values are the no. positive no. tested. PFU = plaque-forming units; ND = not done.
† Virus was simultaneously recovered from plasma (see Results).

XJ Clone 3 (LI > 1.24) (Table 4). In no case did lethality for juvenile mice indicate a significant reversion to virulence.

Serology

Neutralizing antibodies were detected in all macaques inoculated with Candid #1 at some point during the 106-day followup period. In some cases, responses were transient, and detectable only at low serum dilutions (1:8–1:32). Maximum titers ranged from 1:8 to 1:8,192 in individual animals. Antibodies were initially detected on day 14 in two macaques that received the highest vaccine dose (Figure 2). The highest antibody titers were also achieved in animals

TABLE 3

*Identification of isolates recovered from blood of rhesus macaques inoculated with Candid #1 Junin vaccine**

Vaccine dose	Isolate no.	Day of isolation	Log PFU/ml in virus-diluent mixture	Log PFU/ml in virus-serum mixture
16 PFU	626A/M-1	10	5.847	0
	626A/M-2	14	5.911	0
	M377/M-1	14	4.796	0
	M371/M-1	21	5.408	0
318 PFU	M95/M-1	7	4.861	0
	M95/M-2	10	6.336	0
	P658/M-1	4	3.925	0
	P658/P-1†	4	3.859	0
	P658/M-2	7	5.829	1.000
	P658/M-3	10	5.281	0
6,360 PFU	9C94/M-1	4	3.913	0
	T359/M-1	7	5.875	0
	OB27/M-1	10	2.493	0
127,200 PFU	B6973/M-1	7	5.657	0
	B6973/M-2	10	4.812	0
	18081/M-1	1	5.750	0
	18081/M-2	4	4.913	0
	18081/M-3	7	6.517	1.462
	F2/M-1	7	5.041	0
	632B/M-1	4	4.029	0
	632B/M-2	7	5.072	1.097
	632B/P-1†	7	4.348	0
	632B/M-3	10	6.420	0
	364A/M-1	10	5.072	0
	364A/M-2	14	5.009	0.699
	364A/M-3	17	5.560	0
	Candid #1		5.422	0
	XJ Clone 3		5.072	0

* PFU = plaque-forming units.
† Plasma isolate; all others were recovered from peripheral blood mononuclear cells.

TABLE 4

*Virulence of isolates recovered from peripheral blood mononuclear cells of Candid #1–vaccinated rhesus macaques for 11–12-day-old mice**

Vaccine dose	Isolate no.	Day of isolation	PFU/0.02 ml	L.D.$_{50}$/0.02 ml	Lethal index
16 PFU	626A/M-2	14	$10^{3.98}$	$10^{1.90}$	1.98
	M377/M-1	14	$10^{3.14}$	$< 10^{2.00}$	≥4.14
	M371/M-1	21	$10^{4.01}$	$10^{0.00}$	4.01
318 PFU	M95/M-2	10	$10^{3.62}$	$10^{0.37}$	3.25
	P658/M-2	7	$10^{3.80}$	$< 10^{0.00}$	≥4.40
6,360 PFU	T359/M-1	7	$10^{3.43}$	$< 10^{0.14}$	≥4.29
127,200 PFU	B6973/M-1	7	$10^{4.20}$	$< 10^{0.00}$	≥4.20
	18081/M-3	7	$10^{3.49}$	$10^{0.37}$	3.13
	F2/M-1	7	$10^{3.53}$	$10^{0.60}$	2.93
	632B/M-3	10	$10^{3.01}$	$10^{0.19}$	2.82
	364A/M-1	10	$10^{3.50}$	$10^{0.00}$	3.50
	Candid #1		$10^{4.10}$	$10^{0.48}$	3.62
	XJ Clone 3		$10^{6.24}$	$10^{5.00}$	1.24

* PFU = plaque-forming units; L.D.$_{50}$ = 50% lethal dose.

from this group (maximum geometric mean titer [GMT]= 1:1,176 on day 56). Antibodies were not seen until days 17–24 in macaques receiving lower doses, but tended to reach the same maximum level (maximum GMT = 1:84–1:128 on day 106). No seroconversions occurred among placebo recipients.

The time from initial recovery of virus from PBMC to detection of neutralizing antibodies varied from seven to 39 days. However, it should be noted that antibodies were not measured for an entire month following the day 28–bleed. Thus, for the 12 animals in whom virus was recovered early enough to allow for accurate assessment of the interval to seroconversion (i.e., during the first 10–14 days after vaccination), 11 seroconverted 1–2.5 weeks after initial virus recovery.

DISCUSSION

This placebo-controlled study demonstrated that Candid #1, a recently developed live-attenuated Junin virus vaccine, is safe and immunogenic in nonhuman primates. At doses from 16 to 127,200 PFU (a four $\log_{10}$ range), there was no significant effect of immunization on any physical, hematologic, or biochemical parameter measured. Moreover, even at the lowest dose tested, 100% of the animals inoculated responded with detectable neutralizing antibodies.

The infrequent recovery of virus from plasma and the virtual absence of virus in oropharyngeal swabs contrasts sharply with findings in rhesus macaques infected with wild-type Junin virus strains.[10, 11] These results suggest that Candid #1 is relatively limited in its ability to replicate in vivo, and is unlikely to spread among individuals. In previous studies involving wild-type Junin virus strains, cross-contamination of placebo controls housed in the same room was frequently seen.[11](McKee K Jr, unpublished data). No infection of placebo-inoculated macaques occurred in the present experiment.

Candid #1 could be recovered from mononuclear cells in the peripheral circulation for up to three weeks after immunization. While most isolates were made from animals that received the highest vaccine doses, it was possible to recover virus from animals in every dose group. That the viruses recovered were Junin virus is certain; all isolates tested (89% of those recovered) were specifically neutralized by a primate antiserum produced by a single inoculation with Junin virus. This antiserum, although raised to Candid #1 itself, is unlikely to have inappropriately detected an adventitious agent. Candid #1 was prepared and tested in a laboratory suite specifically designated for vaccine production; no other viruses were manipulated in the laboratory during this period. Subsequently, an extensive battery of in vivo and in vitro tests failed to detect any evidence of contamination. An antiserum raised against another Junin virus strain, grown under general laboratory conditions and in the absence of the stringent laboratory controls used in the culture of candidate vaccines, would

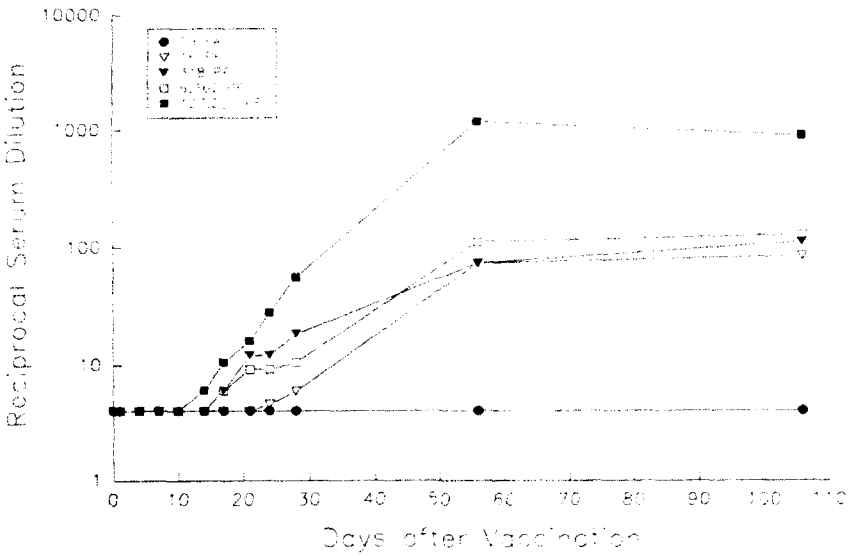

FIGURE 2. Neutralizing antibody response in rhesus macaques inoculated with graded doses of Candid #1 vaccine. Values represent geometric mean titers for animals within each dose group. PFU = plaque-forming units.

have been much more likely to contain antibodies against an undetected adventitious agent. Therefore, use of such a strain could have confused the identity of viruses recovered from animals in these experiments.

Although lethality and disease patterns have been associated with certain wild-type Junin virus strains in animal models, no specific biochemical or antigenic markers for virulence have yet been identified.[10, 18] Thus, assessments of relative virulence among attenuated strains must be made in biological systems of sufficient sensitivity to detect reproducible differences. Previous reports have indicated that 11–14-day-old mice are useful in distinguishing virulent from attenuated Junin virus strains.[9, 14, 15] Typically, lethal indices for wild-type strains in this mouse assay are $\ll 1.00$. In contrast, the previously tested human vaccine candidate, XJ Clone 3, has been repeatedly shown to be significantly more attenuated than wild-type strains (LI 1.25). When we used this system to examine viruses recovered from the blood of Candid #1–vaccinated macaques, we found no evidence of in vivo reversion; all tested isolates (each of which previously had been documented as Junin virus) had an LI in 11–12-day-old mice similar to that of the inoculum (and higher than that for the XJ Clone 3 attenuated control).

Our finding that viremia and neutralizing antibodies did not coexist in any animal suggests that antibody plays a major role in virus clearance. That this is true has been demonstrated in the elegant therapeutic studies of Maiztegui and others[5] and Enria and others,[6] in which human immune plasma containing sufficient neutralizing antibody was administered to patients with AHF within eight days of disease onset, and resulted in prompt clearance of virus and a significant reduction in mortality. However, it is evident that other, as yet undefined, factors are also operative in virus clearance. In the present study, both plasma- and PBMC-associated viremia often disappeared well before the appearance of antibody (up to several weeks). It should also be noted that viremia and circulating antibody frequently coexist in Lassa fever, a related human arenavirus infection, and neutralization of virus using in vitro techniques similar to that for Junin virus is difficult.[19] Clearly, the mechanisms of in vivo neutralization and virus clearance are complex and multi-factorial.

Despite the fact that antibodies were found in every vaccinated animal, the absolute levels observed in some animals were relatively unimpressive. In one macaque vaccinated with 318 PFU, antibodies were detected only on day 56 at a serum dilution of 1:8. Similarly, antibodies were detected only once at a titer of 1:32 in two animals (one each receiving 16 PFU and 318

PFU). In some cases, neutralizing antibodies had reverted to undetectable levels by the time of virulent Junin virus challenge. It should be noted, however, that the sensitivity of the neutralization test used in this study was considerably less (2-4 fold) than that currently available.[20] It is likely, therefore, that the observed serologic response understated that which actually occurred.

These findings extend previous observations that Candid #1 is safe and immunogenic in laboratory animals. Together with previous observations, these studies support the transition of Candid #1 into humans for clinical testing.

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Authors' addresses: Kelly T. McKee Jr. Preventive Medicine Service, Department of the Army, Womack Army Medical Center, Fort Bragg, NC 28307-5000. Julio G. Barrera Oro, Anna I. Kuehne, Joan A. Spisso, and Bill G. Mahlandt, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702-5011.

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